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Evidence for a role of protein phosphatases 1 and 2A during early nephrogenesis

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Evidence for a role of protein phosphatases 1 and 2A during early nephrogenesis. Although most transcriptional events appear to be modulated by reversible protein phosphorylation, little is known about the role of this regulatory system during the development of mammalian organs. Here we have studied the serine/threonine protein phosphatases (PP) 1 and 2A in the early embryonic rat kidney with regard to expression and effects on growth and differentiation. All isoforms of PP-1 and PP-2A were ubiquitously expressed in 15-day embryonic (E15) kidneys (*in situ* hybridization studies). In contrast, mRNA for inhibitor-1 (I-1), an endogenous inhibitor of PP-1, was detected only in undifferentiated stem cells in the outer cortical area. I-1 is a novel marker for these cells. The abundance of the PP-1 protein, confirmed with immunoblotting, was high in the embryonic kidney. In organ culture of E13 kidneys, okadaic acid (OA), an exogenous inhibitor of PP-1 and PP-2A, dose-dependently inhibited growth and nephron formation (apparent half-maximal effect at 6 nM). OA 10 nM had little effect on the growth of cultured E15 kidneys, whereas nephron formation was disturbed and morphological evidence of apoptosis was seen. In summary, this study points towards important roles for protein phosphatases 1 and/or 2A in regulation of mitogenic activity in the early embryonic kidney.

The mammalian kidney is derived from an embryonic structure termed the metanephros. The undifferentiated metanephric mesenchyme is stimulated by the ureteric bud, an outgrowth from the Wolffian duct, to condense and convert into epithelium, thus giving rise to the glomeruli and the proximal and distal tubuli. Various hormones, including IGF-I, IGF-II, TGFα, TGFβ, EGF, HGF and PDGF have been suggested to participate in the sequence of inductive events that lead to the formation of nephrons [1–5], but so far no single factor has been shown to alone promote metanephric development in organ culture [6]. All the above-mentioned hormonal factors will, via membrane receptors, activate one or several intracellular signalling pathways where the final step is a covalent modification of the target protein via phosphorylation catalyzed by protein kinases or dephosphorylation catalyzed by protein phosphatases. The effect on the target protein is often preceded by a series of phosphorylation events that involves both serine/threonine and tyrosine kinases and phosphatases. A recent review article [7] drew attention to the central role that serine/threonine protein phosphatases play for regulation of growth and differentiation. So far, few such studies have been performed on mammalian tissue.

This study was performed to evaluate the role that intracellular signalling via the serine/threonine protein phosphatases 1 and 2A might play for the early development of the kidney. We have determined the distribution of these phosphatases as well as of inhibitor-1, an endogenous inhibitor of PP-1, in the early embryonic rat kidney. We have also examined the effect of okadaic acid, an exogenous inhibitor of PP-1 and -2A, on the growth and differentiation of cultured embryonic rat kidneys.

Methods

Embryos from timed-pregnant Sprague-Dawley rats (Alab, Sollentuna, Sweden) were used. Day 0 of pregnancy was determined by the detection of a vaginal plug. Before removal of the embryos, the rats were sedated with an intraperitoneal injection of either Inactin-Byk (Byk-Gulden, Konstan, Germany) 80 mg/100 g body wt, or sodium pentobarbital (Apoteksbolaget, Stockholm, Sweden) 6 mg/100 g body wt.

Embryonic metanephric kidney rudiments were micro-surgically removed on days 13 or 15 of gestation (E13 or E15). They were dissected free of surrounding tissue, placed on a Nuclepore filter (Costar, Pleasanton, CA, USA) with a pore size of 0.05 μm, and cultured as described [8, 9]. The medium was I-MEM [10] (Gibco, Paisley, Scotland, UK), supplemented with 10% fetal calf serum and 1% L-glutamine. The cultures were kept for four days in a humidified incubator at 37°C with a 95% air/5% CO_2 mixture. The medium was renewed at 48 hour intervals.

Histological sections, 6 μm thick, were cut from cultured kidney explants that had been fixed in Bouin's solution, embedded in paraffin and stained with hematoxylin-eosin.

For DNA quantification, metanephric explants were individually sonicated in a pH 7.4 salt buffer, containing 50 mM $NaHPO_4$ (~ 1 part 50 mM NaH_2PO_4 + 3 parts 50 mM Na_2HPO_4) and 2 M NaCl. Total DNA was quantified with fluorometry as described [11], using a TKO 100 DNA Fluorometer (Hoefer Scientific Instruments, San Francisco, CA, USA) with calf thymus DNA as standard. In all experiments, 8 to 10 kidneys from each category were analyzed. Due to method sensitivity, the very small kidneys belonging to the E13 category were pooled (2 kidneys/tube) and analyzed together. These readings were accordingly divided by a factor of 2. Readings were always compared with controls (% of

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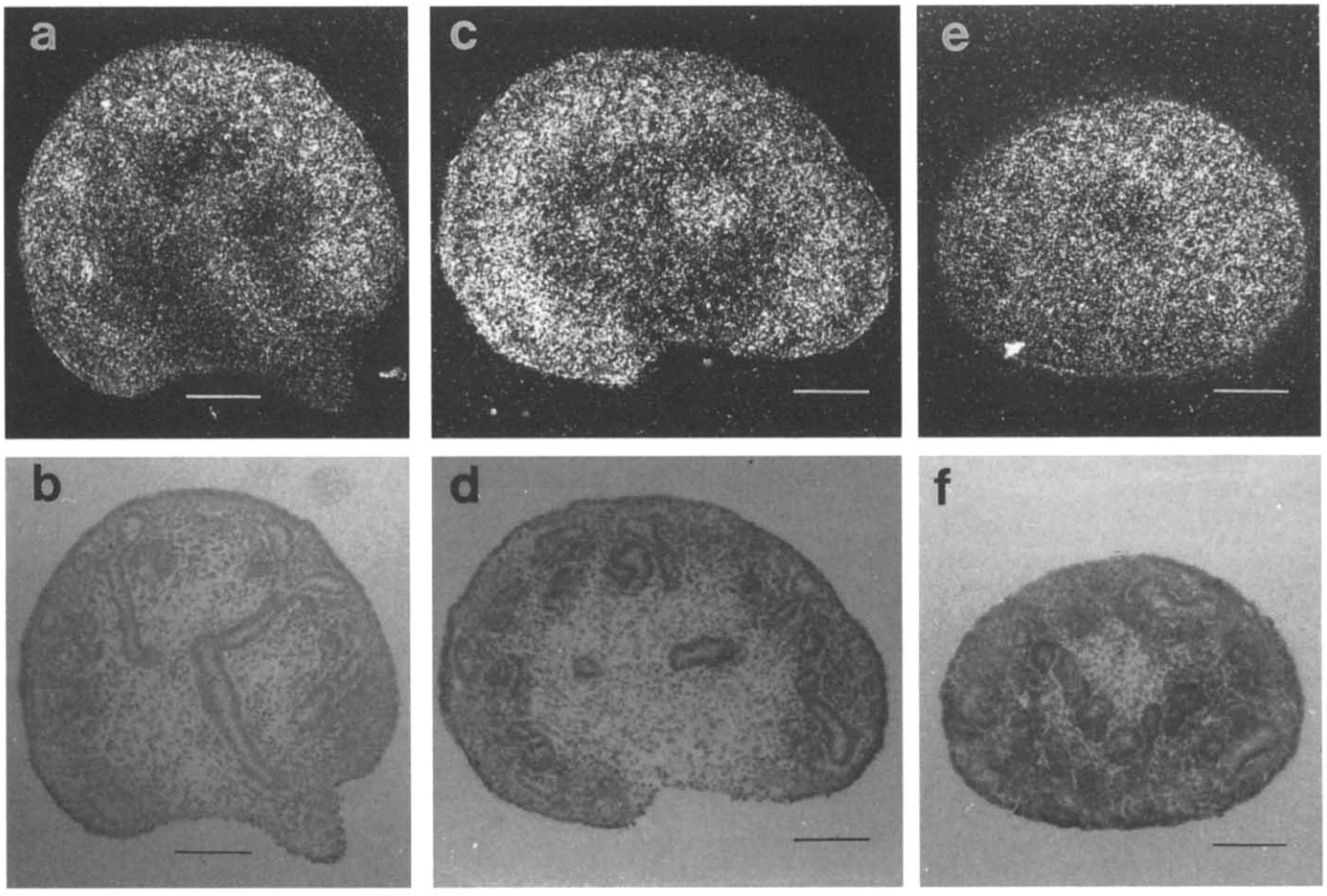

Fig. 1. *Dark field (***a**, **c**, **e***) and bright field (***b**, **d**, **f***) micrographs of in situ hybridizations for PP-1 mRNAs in E15 kidneys.* Signals for PP-1α (a, b), PP-1β (c, d) and PP-1γ (e, f) are all abundant and uniformly distributed throughout the fetal kidneys. Magnification is 50×. Bar = 200 μm.

control). When readings from consecutive experiments were used in the same graph, they were first standardized versus their respective controls. Student's *t*-test was used for statistical analysis, and P values < 0.05 were considered significant.

In situ hybridization

In situ hybridization was performed according to Ernfors et al [12], with a few modifications. E15 rat kidneys were embedded in Tissue-Tek (Miles Inc., Elkhart, IN, USA), frozen in liquid nitrogen, cut in 10 μm sections on a cryostat (−20°C) and thawed onto slides pre-treated with poly-L-lysine (Sigma Chemical Co., St. Louis, MO, USA) (50 mg/ml). The sections were fixed in 4% paraformaldehyde, rinsed twice in phosphate-buffered saline, dehydrated in a graded ethanol series including a five minute incubation in chloroform. The following oligonucleotides were used as hybridization probes: for rat protein phosphatase 1α mRNA 5′-GAG AGG CCA GTG TGA CCC ATG GCA GCA TGA TTT CTG TAC A-3′; rat protein phosphatase 1β mRNA 5′-AGC ACC CAA TAC TCA GAG CTG ACA ATG TCC CAC TGA CCA G-3′; rat protein phosphatase $1\gamma_1$ mRNA 5′-TGT GAG TTC TGT ATA AAC CGG TGG ACG GCA AGT TAG TTC C-3′; rat protein phosphatase 2Aα mRNA 5′-CGC TAT GCC AGA AAC TGG ATT CTT GAC CAA CAA TGT CGC GTC AGC-3′; rat protein phosphatase 2Aβ mRNA 5′-ACC CTT TCC CTT AAC CAG TCA CGA AGG ACG GCT ACT ACA GCA GGC-3′; rat inhibitor-1 mRNA 5′-CCA GTG TCC ATG AAC TTC CAC ACT CAC TGG CGA TCC CCG GAT GTC-3′. All the oligonucleotides are antisense and therefore complementary to the mRNAs for which they are specific. Using terminal deoxyribonucleotidyl transferase (Scandinavian Diagnostic Services, Falkenberg, Sweden), the probes were labeled at the 3′ end with [α-^{35}S]dATP (Amersham, Little Chalfont, Buckinghamshire, UK) to a specific activity of 10^9 cpm/mg. Hybridization was performed in 50% formamide, 4x SSC (0.6 M sodium chloride, 0.06 M sodium citrate, pH 7.0), 1x Denhardt's solution, 10% dextran sulfate, 0.25 mg/ml yeast tRNA, 0.5 mg/ml sheared salmon sperm DNA, 1% sarcosyl, 0.02 M Na_2HPO_4/NaH_2PO_4 (pH 7.0), 0.05 M dithiothreiotol, using 10^7 cpm/ml for each probe. The sections were hybridized at 42°C for 15 to 18 hours in a humidified chamber with 0.1 ml of hybridization solution per slide. They were subsequently washed 4 × 15 minutes at 56°C in 1x SSC. Controls for the specificity of the *in situ* labeling were demonstrated by a concentration-dependent depletion of the signal, provided by a 50- to 100-fold excess of unlabeled probe added to the hybridization cocktail. Slides were dipped in Kodak NTB-2 photo emulsion (diluted 1:1 in water), exposed for three to

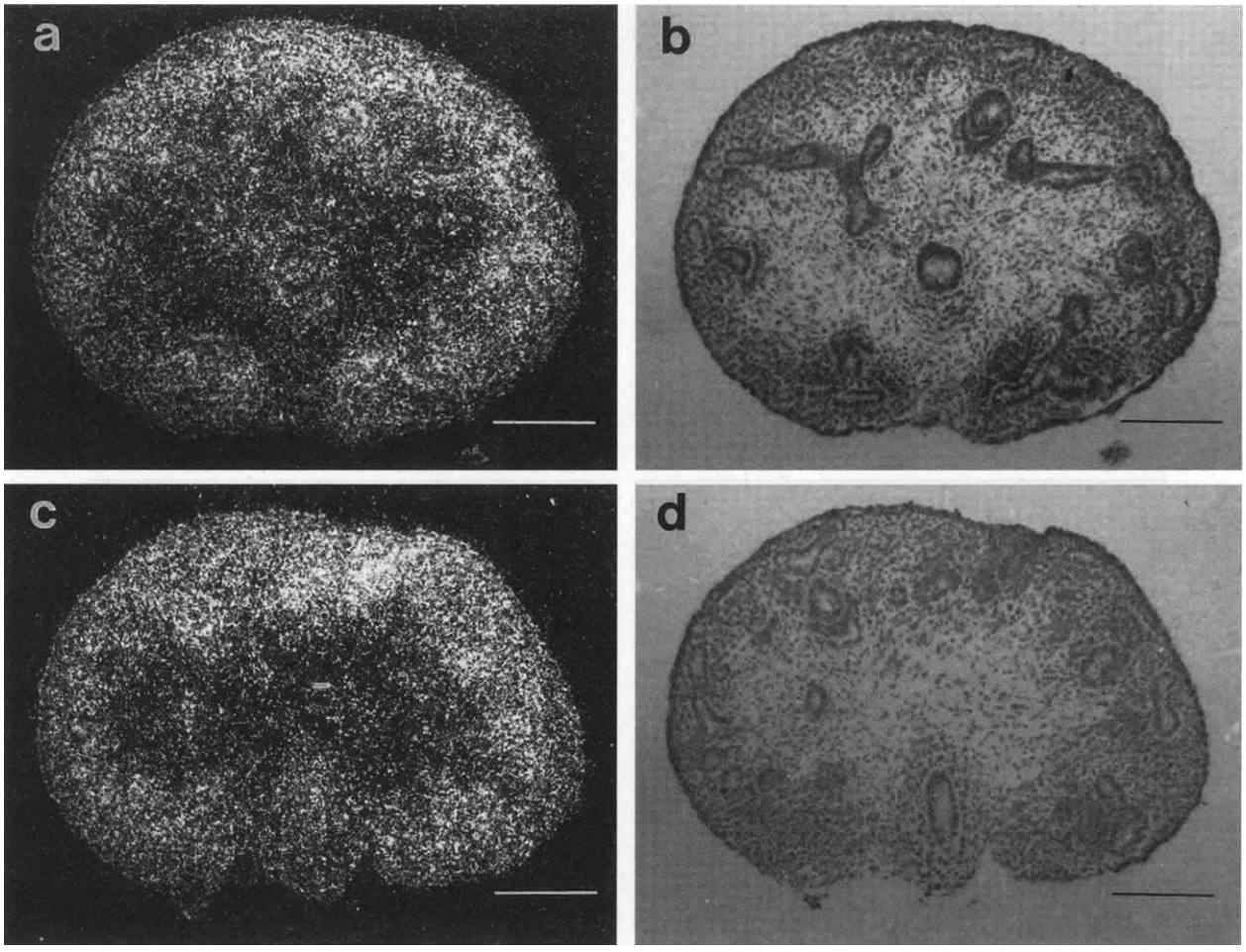

Fig. 2. *Dark field (a, c) and bright field (b, d) micrographs of in situ hybridizations for PP-2A mRNAs in E15 kidneys.* PP-2Aα (a, b) and PP-2Aβ (c, d) are abundantly expressed and uniformly distributed throughout the fetal kidneys. Magnification is 67×. Bar = 200 μm.

five weeks at 4°C, developed, fixed and counterstained with cresyl violet. Photographs were taken under bright field and dark field illumination, using Kodak T-MAX 400 film.

Immunoblotting

Adult (40-day-old) renal medulla, E13 and E15 metanephric mesenchymes were sonicated in 1% sodium dodecyl sulfate with a Branson Sonifier B-12 (Branson Power Company, Danbury, CT, USA). Total protein was determined with a protein assay (Bio-Rad, Hercules, CA, USA), using bovine serum albumin as a standard. Protein (4 mg per lane) was loaded on a 12% acrylamide mini-gel, separated with electrophoresis and transferred electrophoretically to nitrocellulose. Rabbit antisera raised against synthetic peptides from the C-terminals of PP-1α and PP-1γ_1 catalytic subunits were used to identify protein phosphatase 1α and γ (apparent molecular wt 37 kDa), respectively. The specificity of these antibodies has previously been demonstrated [da Cruz e Silva EF and Greengard P, unpublished observations; 13]. Detection of the bound primary antibody was performed with an alkaline phosphatase system (Promega, Madison, WN, USA).

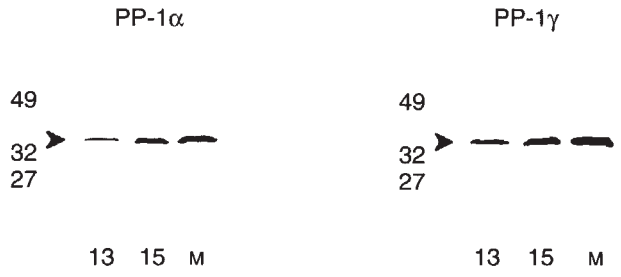

Fig. 3. *Immunoblot for PP-1 catalytic subunits α and γ in E13 and E15 kidneys ("13" and "15", respectively), compared with adult renal medulla ("M").* Both isoforms are detected as bands corresponding to an apparent molecular wt of approximately 37 kDa. Note that the abundance of both isoforms increases with age and that adult levels for PP-1α appear to be reached at the E15 stage.

Drugs used

In some of the protocols, the kidneys were cultured in the presence of okadaic acid (Calbiochem, La Jolla, CA, USA), an inhibitor of PP-1 and PP-2A [14]. Okadaic acid inhibits purified

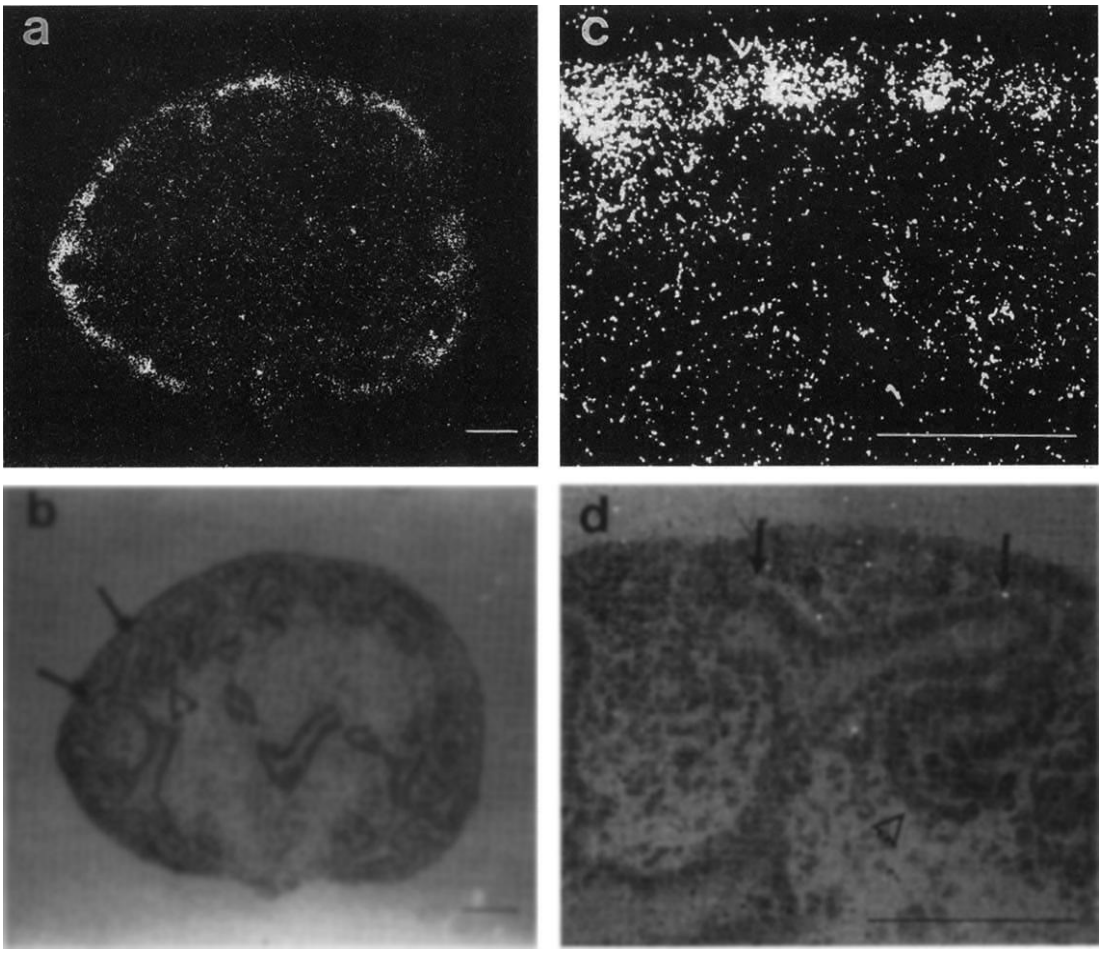

Fig. 4. *Dark field (***a**, **c***) and corresponding bright field (***b**, **d***) micrographs of in situ hybridizations for Inhibitor-1 (I-1) mRNA in E15 kidney.* a, b. I-1 labeling is detected almost exclusively in the outermost part of the fetal kidney, in mesenchymal cells with no morphological signs of induction. c, d. Detail magnification reveals that ureteric epithelium and mesenchymal condensations is almost devoid of signal. Note that the strongest expression is seen in cells lying on the cortical side of the ureteric bud (the tips of which are marked with arrows), where no induction takes place. An adjacent S-shaped body is also seen (open arrow). Magnification is 67× (a, c) and 270× (b, d), respectively. Bar = 100 μm.

PP-2A about 100-fold more potently than it inhibits purified PP-1 [14–16]. Okadaic acid was dissolved in dimethyl sulphoxide (DMSO) (Fluka Chemie AG, Buchs, Switzerland) and added to the medium at onset of culture. Equivalent amounts of DMSO were added to the controls.

Results

The catalytic subunit of PP-1 is encoded by three different genes (α, β and γ) and that of PP-2A by at least two different genes (α and β). The expression of PP-1 and PP-2A mRNA in the E15 kidney (which contains uninduced mesenchymal cells as well as S-shaped bodies and tubules) was studied with *in situ* hybridization technique. Strong signals for the mRNA for both phosphatases were detected in uninduced and condensing mesenchyme, comma-shaped and S-shaped bodies, ureteric bud and stroma. The intensity was approximately the same in all cell types (Figs. 1 and 2).

The presence of the PP-1 protein in E13, E15 and adult kidneys was confirmed with immunoblotting using antibodies raised against PP-1α and γ_1. PP-1α and γ_1 were both present in the embryonic kidneys. The abundance was higher in E15 than in E13 kidneys. The abundance of PP-1α was similar in E15 kidneys and adult medulla, whereas the abundance of PP-1γ_1 appeared to be slightly lower in E15 kidneys than in adult medulla (Fig. 3). In the adult kidney, tissue from the outer medulla was used, since this tissue has a very high content of PP-1.

Inhibitor-1 (I-1) is an endogenous inhibitor of PP-1. *In situ* hybridization for I-1 mRNA was performed in E15 kidneys. In contrast to the ubiquitous localization of PP-1 and PP-2A, mRNA for I-1 was only detected in mesenchymal cells in the outer cortical area of the developing kidney (Fig. 4). These cells have not yet been reached by the ureteric bud and have thus remained metanephric mesenchymal stem cells.

Organ culture of embryonic kidneys is an excellent and well-defined model system for the *in vitro* study of early mesenchymal differentiation [8, 17], closely resembling the development *in vivo*. During the first 24 hours of culture, the early embryonic kidneys do not grow much, presumably because they need to adapt to the culture situation. Therefore, to evaluate the functional role of PP-1 and PP-2A during early kidney development, okadaic acid

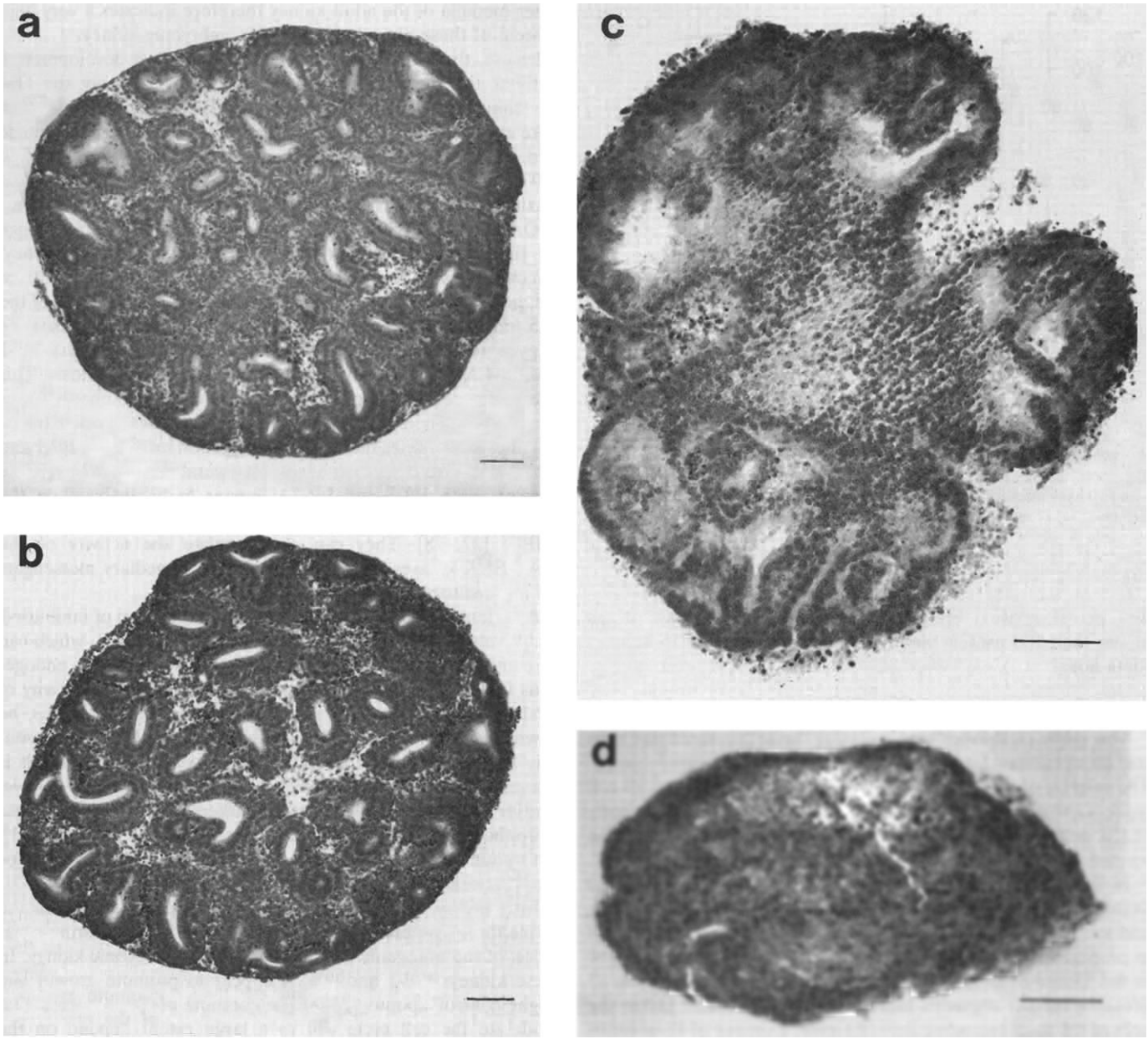

Fig. 5. *Hematoxylin-eosin-stained sections of E13 kidneys after four days of culture with or without OA.* **a**, Control; **b**, OA 3 nM; **c**, OA 6 nM; **d**, OA 10 nM. Morphogenesis and growth are normal in a and b, but are severely disturbed in c and d. Magnification is 67× (a, b) and 135× (c, d), respectively. Bar = 100 μm.

(OA), a potent inhibitor of both phosphatases, was added to organ cultures of E13 and E15 kidneys during four days of culture. After two days, the okadaic acid-treated kidneys started to be distinguishable from control kidneys. After four days, OA was found to dose-dependently inhibit growth of the E13 kidneys. Apparent half-maximal effect was reached at 6 nM and full growth inhibition at 10 nM. OA 6 nM also caused a marked disturbance of tubule formation (Fig. 5). To evaluate whether the observed growth inhibition was due to a true reduction in cell number rather than a decrease in cell size, we quantified the DNA content per cultured E13 kidney. DNA analysis revealed a markedly lower total DNA content, with half-maximal effect at 6 nM and full effect at 10 nM OA (Fig. 6). In this assay, OA in doses of 1 to 10 nM neither affected the explant size nor the amount of DNA (Fig. 6) of E15 kidneys cultured for four days. Yet, OA 10 nM severely affected nephron formation. The central parts of OA-treated E15 kidneys had a larger fraction of stromal tissue and fewer epithelial structures than the controls. They also displayed a higher degree of condensed or pycnotic nuclei in the mesenchymal parts (Fig. 7d), suggesting apoptosis [18, 19]. The tubules, comma- and

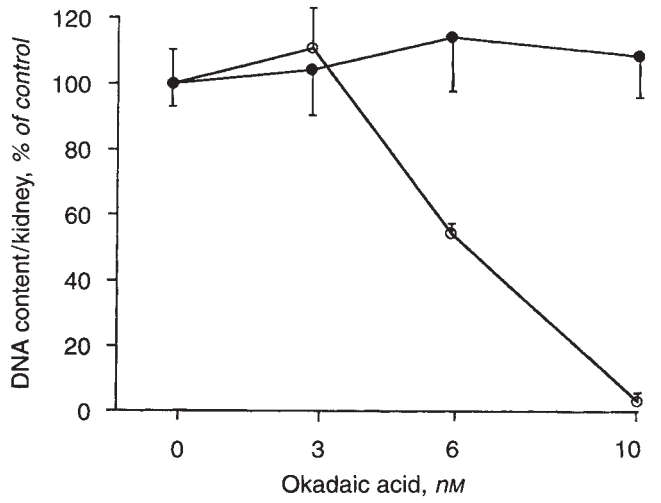

Fig. 6. *Total DNA content (expressed as % of control) in E13 (○) and E15 (●) kidneys cultured for four days in the presence of OA.* Values are mean plus or minus SE. For each category, 8 to 10 kidneys were analyzed. The 6 and 10 nM values for E13 kidneys are highly significant ($P < 0.01$). In the E15 category, there is a slight, but not significant, increase of DNA at 6 nM.

S-shaped structures were poorly organized (Fig. 7). OA in doses higher than 100 nM completely inhibited growth of E15 kidneys (data not shown).

Discussion

This study establishes vital roles for the serine-threonine protein phosphatases 1 and/or 2A during early kidney development. The mRNA of these phosphatases was well expressed in all cell types. Low doses of okadaic acid inhibited growth in the early (E13) embryonic kidney and caused disturbances of nephron formation in E15 kidneys.

In a previous study the distribution of PP-1 mRNA was examined with *in situ* hybridization technique in the adult kidney and was found to be differentially distributed [Li et al, manuscript in preparation; 20]. The signal for all PP-1 isoforms was very low in the S1 and S2 proximal tubular segments, but strong in the S3 proximal tubular segments located in the inner cortex and in the cells of the thick ascending limb of Henle. Labeling of all isoforms was also strong in the ureter, the former inducer. In contrast, in the embryonic kidney the mRNAs for PP-1α, β and γ were evenly distributed in all cells. This developmental change in the distribution suggests that PP-1 plays a different role in the developing and the mature kidney. The appearance of the mRNA for PP-1 isoforms in all cells in the embryonic kidney further indicates that these enzymes may be of importance both for induction and for early differentiation and growth. In line with this, in ongoing studies we (JS) have observed that the abundance of PP-2A mRNA is much lower in the adult than in the embryonic kidney.

Both the α and the γ isoform of PP-1 appeared to increase in abundance from E13 to E15 kidneys. As a reference tissue, the outer medulla from rat kidney was used. In this tissue the abundance of PP-1α and γ_1 is comparable to that of the brain and much higher than in most other tissues. The finding that the abundance of PP-1α and γ_1 was similar in E15 kidney and in the outer medulla of the adult kidney therefore indicates a very high content of these phosphatases in the embryonic kidney.

In rats, the E13 kidney represents the earliest developmental state of the metanephric kidney. At this stage only the first branching of the ureter has occurred. These embryonic kidneys were extremely sensitive to the growth retarding effect of okadaic acid. OA inhibits purified PP-1 and -2A with IC_{50} values of 15 to 200 nM and 0,5–1,0 nM, respectively [15]. Since in E13 kidneys half maximal effect on DNA content was observed with less than 10 nM of OA, it is possible that PP-2A might be of particular importance for the growth of the early embryonic kidney. In E15 kidney, somewhat higher doses were required to inhibit growth. OA 10 nM did, however, profoundly affect the structural development of the E15 kidney. Of particular interest was the increased appearance of pycnotic nuclei in the OA-treated kidney, indicating that PP-1 and/or -2A might be involved in the control of apoptosis. This does not necessarily imply a specific role for protein phosphatases in apoptosis. There is ample evidence from studies of cell extracts, cell lines and oocytes that dephosphorylation induced by PP-1 and PP-2A can have significant effects on several steps in the cell cycle [21–26]. Both PP-1 and PP-2A appear to be involved in the regulation of the activity of the maturation-promoting factor (MPF) [27, 28]. They can also regulate the activity of the MAP-ERK kinases [29, 30], important intermediary messengers for most mitogens.

A striking finding in this study was the expression of inhibitor-1 exclusively in the mesenchymal stem cells. Inhibitor-1, which can be activated by PKA-dependent phosphorylation, is an endogenous inhibitor of PP-1. This finding indicates that the activity of PP-1 in the mesenchymal stem cells will, at certain stages, be down-regulated by a signalling system where cAMP is a messenger. The localization of inhibitor-1 in mesenchymal stem cells is interesting from another aspect, as it provides us with a novel marker for these cells. A variety of markers for the different stages of epithelial and stromal differentiation have been identified [31], but to our knowledge inhibitor-1 is the first described marker for the stem cells.

In summary, this study points towards important roles for the serine/threonine protein phosphatases 1 and/or 2A during regulation of the mitogenic activity in the early embryonic kidney. In these kidneys, PP-1 and/or -2A appear to promote growth and might control apoptosis. The net activity of the proteins that modulate the cell cycle will to a large extent depend on the proportion of the phosphorylated and dephosphorylated state of these proteins, which in turn depends on the activity of protein kinases and phosphatases. Further studies on PP-1 and -2A activity and their relationship to serine/threonine kinase activity might therefore provide a better understanding of the mechanisms that lead to kidney deformities and the formation of kidney tumors.

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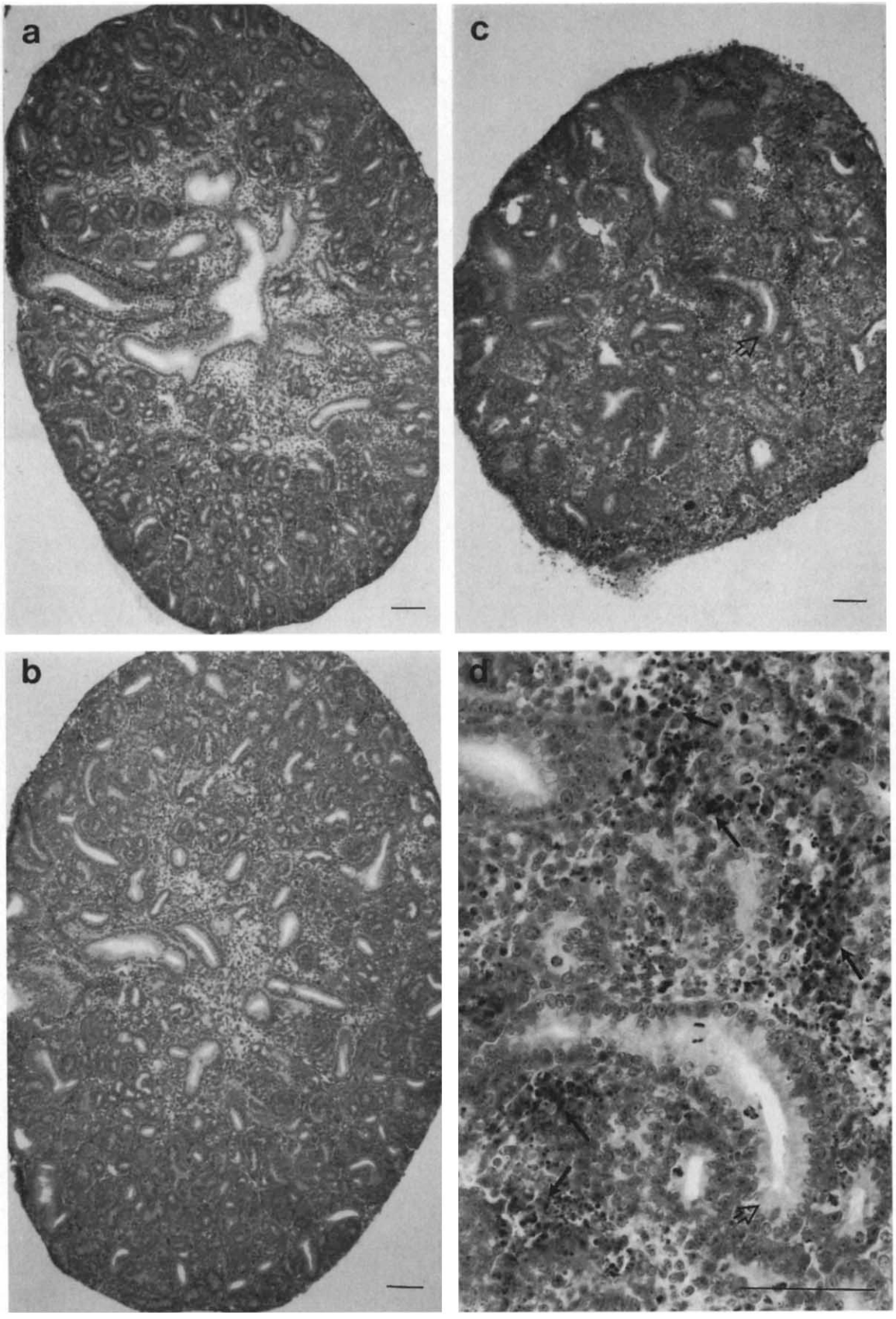

Fig. 7. *Hematoxylin-eosin-stained sections of E15 kidneys after four days of culture with or without OA.* **a**, Control; **b**, OA 6 nM; **c**, OA 10 nM; **d**, OA 10 nM, detail magnification. Growth is similar in control kidneys and OA-treated kidneys. However, at 10 nM, morphogenesis is disturbed; ureteric branching and tubular epithelium are more sparse, and instead an increased amount of stromal tissue can be seen. Note groups of pycnotic and fragmented nuclei in the mesenchyme (arrows). The tip of a ureteric bud (same in c and d) is marked with an open arrow. Magnification is 67× (a, b, c) and 270× (d), respectively. Bar = 100 μm.

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